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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Eric A. JOHNSON et al.

Appln. No. 08/458,019 Group Art Unit: 1808

Filed: 1 June 1996 Examiner: H. Lilling

For: **PROCESSES FOR IN VIVO PRODUCTION OF ASTAXANTHIN AND PHAFFIA RHODOZYMA YEAST OF ENHANCED ASTAXANTHIN CONTENT**

DECLARATION UNDER 37 C.F.R. § 1.132

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BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Stephen Hiu, do hereby declare and state:

I hold a Ph.D. degree.

I am President of IGENE Biotechnology, Inc., assignee of the above-referenced application by virtue of an Assignment recorded at Reel 4952, Frame 0724.

I reviewed the Office Action of 13 November 1996 wherein the Examiner disputed the reproducibility of the instant invention and hence enablement of the claimed invention, and thus rejected claims 25-34 under 35 U.S.C. § 112, first paragraph.

In response thereto, I reviewed the laboratory records of the instant inventors or of technicians who worked under the direction and supervision of the instant inventors to provide the following summary of data which establishes the reproducibility of the methods taught in the instant application and the sufficient enablement of the claimed invention.

To provide a proper frame of reference, it should be noted that the strains set forth in the instant application are coded. Referring to instant Figure 2, the following Table provides the synonymous original strain and patent strain designations.

Original Designation	Patent Designation
Ant-1	IGI887J0
Ant-1-4	IGI887J2
104K	IGI887J3
104K2	IGI887J4
K2	IGI1287J1
K20	IGI887JI
Ant-1-460	IGI2880B60

Experiment 1

Strain Ant-1-4 (IGI887J2) was treated with nitrosoguanidine (NTG) and plated onto YM media as taught in the instant application. Approximately 600 yeast were plated per petri plate.

The plates were assessed visually for enhanced pigment level. The individual colonies were cloned and amplified. The individual colonies were grown in YM broth as described in the instant application.

Then, the level of astaxanthin was determined for the individual isolates.

Amount of Astaxanthin per Gram Dry Yeast[1]	Number of Strains
700-800 ppm	2
800-900 ppm	3
900-1000 ppm	2

Experiment 2

Strain Ant-1-4 was treated as in Experiment 1. The following obtained stains were within the scope of the claimed invention:

Amount of Astaxanthin per Gram Dry Yeast	Number of Strains
600-700 ppm	1
700-800 ppm	2
800-900 ppm	9
900-1000 ppm	5
1000-1200 ppm	1
> 1200 ppm	1

Experiment 3

Strain Ant-1-460 was treated as in Experiment 1 and assessed for astaxanthin content with the following results:

Amount of Astaxanthin per Gram Dry Yeast	Number of Strains
900-1000 ppm	1
1000-1100 ppm	5
1100-1200 ppm	3
1200-1300 ppm	1
1300-1400 ppm	1

[1] μ/g dry yeast = 1 part per million (ppm)

Experiment 4

Strain Ant-1-460 was treated with NTG as in Experiment 1 with the following results:

Amount of Astaxanthin per Gram Dry Yeast	Number of Strains
< 1000 ppm	4
1000-1100 ppm	3
1100-1200 ppm	10
1200-1300 ppm	6
1300-1400 ppm	5
1400-1500 ppm	14
1500-1600 ppm	8
1600-1700 ppm	5
1700-1800 ppm	1
1800-1900 ppm	4
1900-2000 ppm	1
> 2000 ppm	4

Experiment 5

A highly pigmented strain obtained from Ant-1-4 was irradiated with UV light as in Example 1 of the instant application and plated on YM plates. Colonies with enhanced pigmentation were selected and amplified in YM medium.

Amount of Astaxanthin per Gram Dry Yeast	Number of Strains
700-800 ppm	3
800-900 ppm	1
900-1000 ppm	6

1000-1100 ppm	2
1220-1300 ppm	1
1300-1400 ppm	4
1400-1500 ppm	1
1500-1600 ppm	1
1600-1700 ppm	2
> 1700 ppm	1

Experiment 6

Wild-type Phaffia ATCC 24202 were selected on YM agar containing 8 μg/ml tunicamycin as taught in the instant application. Of those colonies that survived the selection, one produced 611 ppm astaxanthin in YM broth compared to 269 ppm of the parent wild-type strain.

Experiment 7

Wild-type Phaffia ATCC 24202, grown on YM media, were exposed to 5 mM mevalonic acid lactone as taught in the instant application. The amount of mM mevalonic acid lactone as taught in the instant application. the amount of mevalonic acid lactone was increased to 25 mM mevalonic acid lactone. The parent strain produced 227 ppm astaxanthin. Three strains which were selected from the 25 mM plates were obtained and found to produce more astaxanthin than the parent strain. For example, one strain mev6 produced 627 ppm astaxanthin.

Clearly, the instant invention teaches a reproducible process for obtaining yeast within the scope of the instant application. In each experiment, yeast within the scope of the claimed invention were obtained.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

11/7/97
Date

Stephen Hiu